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Cytogenetical and biochemical characterization of a dG + dC-rich satellite DNA in the primate *Cebus capucinus*.

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Résumé.

Un DNA satellite très abondant et riche en dG+dC du primate Cebus capucinus *a été caractérisé dans ses propriétés cytogénétiques et biochimiques dans le but d'étudier la corrélation entre les propriétés de coloration de l'hétérochromatine et la composition en base du DNA très répétitif correspondant.*

Les techniques de coloration, ainsi que l'incorporation d'un analogue de base, montrent que les segments hétérochromatiques des chromosomes de C. capucinus *correspondent à un satellite riche en dG+dC. Ce satellite a été détecté et isolé par centrifugation en gradient de densité, marqué radioactivement et localisé par hybridation* in situ *sur les segments hétérochromatiques.*

Mots-clés : DNA satellite de primate / coloration de l'hétérochromatine / hybridation *in situ*.

Summary.

A very abundant and dG+dC rich DNA satellite from primate Cebus capucinus *has been characterized in its cytogenetic and biochemical properties wit the purpose of studying the correlation between the staining properties of heterochromatin and the base composition of the corresponding very repetitive DNA.*

The staining techniques, as well as incorporation of base analogues, show that the heterochromatin segments of C. capucinus *chromosomes correspond to a dG+dC-rich satellite. This statellite was detected and isolated by centrifugation in density gradient, radioactively labelled and localized by* in situ *hybridization on heterochromatin segments.*

Key-words : DNA satellite of primate / staining properties of heterochromatin / *in situ* hybridization.

Introduction.

A number of investigations have shown that constitutive heterochromatin, namely the heavily stained chromosomes segments seen in C-band preparations, contains highly repetitive DNA sequences [see ref. **1** for a review]. These sequences may, however, be very different in base composition, as shown by studies in which C-banding was accompanied by the isolation and characterization of highly repetitive DNA sequences and by *in situ* hybridization [**2-9**]. A number of other cytogenetic staining techniques have been applied to constitutive heterochromatin like quinacrine mustard

Abbreviations :

BAMD, 3,6-bis (acetato-mercurimethyl) dioxane ; r_f, molar ratio of BAMD to DNA phosphate ; SSC, standard saline citrate solution, (0.15 M NaCl, 0.015 M Na citrate, pH 7.2).

[10], Hoechst 33258 [11], T-banding [12], Giemsa 11 [13]. For some heterochromatin regions stained by these techniques, it has been possible to identify the corresponding DNA [14, 15] and to investigate the correlation between staining properties of heterochromatin and base composition of the corresponding DNA [16-21]. Such studies have indicated a general relationship between dA + dT-richness and Q- or G-positive bands, on one hand, and between dG + dC-richness and R- or T-positive bands, on the other hand [see ref. 22 for a review]. Exceptions to this rule have been found, however, as well as situations where it is difficult to establish the relationship because of the short length of heterochromatic segments and/or because of the heterogeneous staining of the segments. In the case of human chromosomes, for example, the main heterochromatic regions are both R- and Q-negative, but can be stained by certain G-banding techniques. While in these cases no obvious solution is available, it is clear that further investigations may help in establishing cytogenetic criteria for a correct assessment of the base composition of heterochromatins.

Having found in the chromosomes of a primate, *Cebus capucinus* (*Platyrrhini, Ceboidae*), long heterochromatic segments representing 10-12 per cent of the overall chrosomose length, and endowed with well-defined R- and T-positive staining properties [12], we thought that this system was ideally suited to test the correlation between staining properties and base compositions of the corresponding DNA. We first characterized the heterochromatic segments by labelling techniques, which indicated a dG + dC richness in them. We then detected and isolated a dG + dC-rich satellite by density gradient centrifugation of *C. capucinus* DNA. Finally, we have shown that the isolated labelled satellite DNA does, indeed, hybridize *in situ* on the heterochromatic segments.

Materials and Methods.

Animals and Tissues.

Two specimens of *C. capucinus* were obtained from the Museum National d'Histoire Naturelle, Paris, France. The liver of one specimen was used for DNA extraction. Lymphocytes and fibroblast cultures were used for cytogenetic analysis ; fibroblasts for *in situ* hybridization experiments.

Chromosome staining.

The techniques used were : R-banding [23], T-banding [24], Q-banding [10], and C-banding [25]. 5-bromodeoxyuridine (BrdU) incorporation (10 µg/ml of medium) was carried out during one (15 hours), two (30 hours) or three (45 hours) cell cycles. Preparations were stained with acridine orange [26].

DNA preparation, fractionation and analysis.

C. capucinus DNA was prepared according to Filipski *et al.* [27]. The molecular weight of the DNA preparation was estimated by gel electrophoresis to be close to 20.10^6. DNA was fractionated by preparative centrifugation in BAMD/Cs_2SO_4 density gradient at pH 9.2 [28, 29] using an r_f value (the BAMD/nucleotide molar ratio) of 0.12 ; this choice was made on the basis of analytical density gradient experiments. The fractions collected were examined by analytical CsCl density gradient centrifugation.

Restriction enzyme degradations of *C. capucinus* DNA, and gel electrophoresis of restriction fragments were done as described previously [30]. DNA P^{32}-labelling by nick-translation was done as described by Jeffreys [31]. Other techniques were described elsewhere [32].

In situ *hybridization.*

The technique used was adapted from Gall and Pardue [33], omitting the RNase step. BrdU (10 µg/ml) was introduced in the cell culture medium for 7 hours before cell harvesting, in order to identify the chromosomes on the basis of the induced banding after acridine orange staining and to facilitate denaturation in the heat-resistant R-positive heterochromatic regions (see Results). Preparations were observed under ultraviolet light. Well-spread metaphasic plates were photographed and their position on the slides noted. Preparations were then put in Earle's balanced salt solution [34], pH 6.5, at 87° for 30 seconds. This denaturing treatment deserves some comments. We had observed that previous BrdU incorporation followed by fluorochrome staining and ultraviolet irradiation greatly sensitized chromosomes to heat denaturation, so that preparations treated as described above gave the same results as preparations heated for 90 minutes [34]. In addition, the described treatment allows the denaturation of otherwise very resistant (T-positive) regions and the selective denaturation of regions which incorporate BrdU. Preparations were then rinsed with distilled water and with 70 per cent, 90 per cent and 99 per cent ethanol.

After addition of 40 µl of the ^{32}P-labelled *C. capucinus* DNA, (containing a total of 14.10^6 or 3.10^6 cpm), each slide was covered with a 22 × 22 mm coverslip and incubated in a moist chamber for 15 hours at 65°. Preparations were extensively rinsed with hot (65°) 3 × SSC and 2 × SSC containing Denhardt's buffer [35] and with 50 per cent, 70 per cent, 90 per cent and 99 per cent ethanol ; SSC is a standard saline citrate solution (0.15 M NaCl, 0.015 M Na citrate, pH 7.2).

Autoradiography was performed with Kodak AR10 stripping film. Slides were stored in the dark at 4° for 5 or 20 days according the amounts of radioactive DNA probe used. Development was made with Kodak D19 for 4 min at 20° and fixation with Kodak Unifix for 3 min. Preparations were then extensively rinsed with tap water and stained with 10 per cent Giemsa, but contrast was poor.

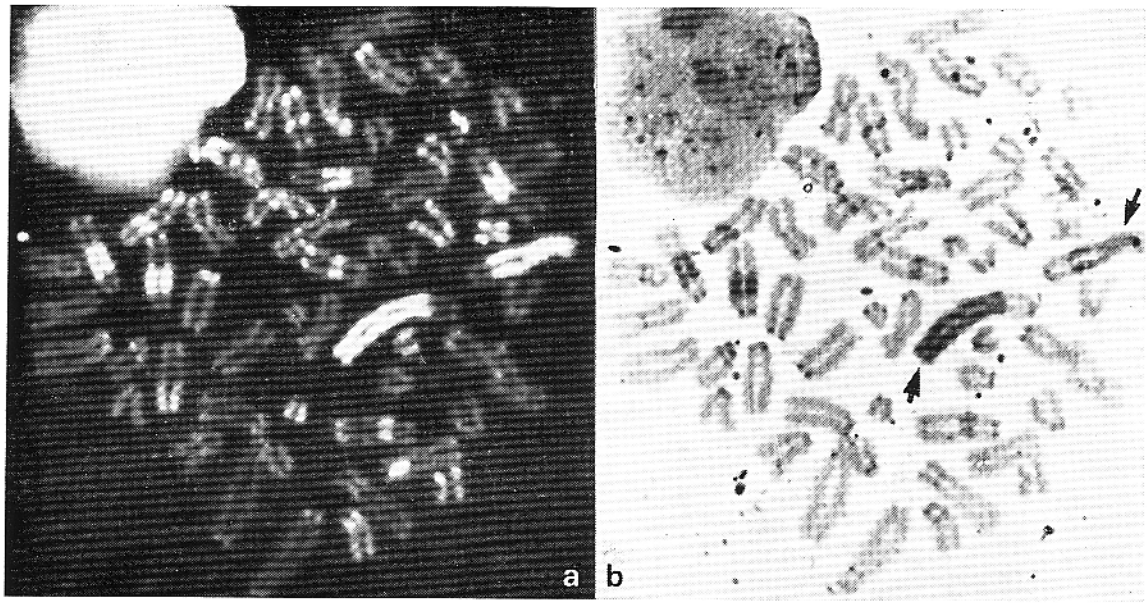

FIG. 1. — *Sequential staining of metaphase chromosomes of* C. capucinus.

(a) T-banding after acridine orange staining, showing positive staining of the large segments of non-centromeric heterochromatin and of euchromatic T-bands.

(b) C-banding after Giemsa staining of the same chromosomes, showing staining of all heterochromatic regions and particularly of the previously T-positive large segments. Chromosomes 9 (arrows) carry a particularly long heterochromatic segment. Euchromatic T-bands are unstained.

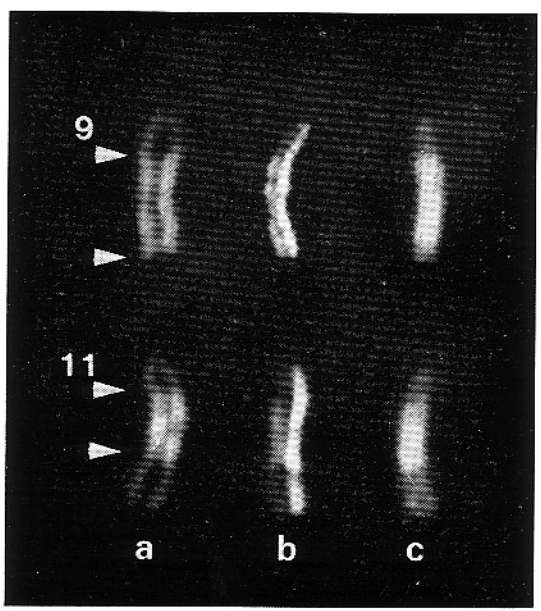

FIG. 2. — *BrdU-incorporation in chromosomes 9 and 11 of* C. capucinus.

Acridine orange staining. Incorporation occurred during :

(a) the whole last S-phase ;

(b) the two last S-phases ;

(c) the three last S-phases.

Heterochromatic segments (arrows) always appears relatively brighter than euchromatin and are thus less substituted by BrdU.

Results.

Cytogenetic characterization of C. capucinus : *heterochromatin banding techniques.*

The R-banding showed, in addition to the R-positive and R-negative euchromatic bands, long segments characterized by a homogeneous intense staining : such segments were particularly long on chromosome 9, but were also present on chromosomes 10, 11, 12, 13, 18 and 19 [12]. After T-banding and acridine orange staining, these segments have a very bright appearance (fig. 1a). After Q-banding, they exhibit a dull fluorescence. C-banding showed that the same segments were intensely stained, as was the juxtra-centromeric heterochromatin (fig. 1b). These segments can therefore be classified as constitutive heterochromatin.

BrdU incorporation.

The incorporation during the whole last S-phase (15 hour treatment) followed by acridine orange staining showed that all the euchromatin was dark and reddish, whereas the heterochromatin appeared relatively bright (fig. 2). After BrdU incorporation during two consecutive S-phases (30 hour treatment), the well-known fluorescence asymmetry of sister chromatids was found on all chromosomes, but this asymmetry was less obvious in the heterochromatin regions because the less fluorescent chromatids were relatively too bright at these places. After BrdU incorporation during 3 consecutive S-phases (48 hour treatment), chromosomes showing fluorescence asymmetry exhibited the same appearance as those having incorporated BrdU during two S-phases. Chromosomes showing no asymmetry (having incorporated BrdU

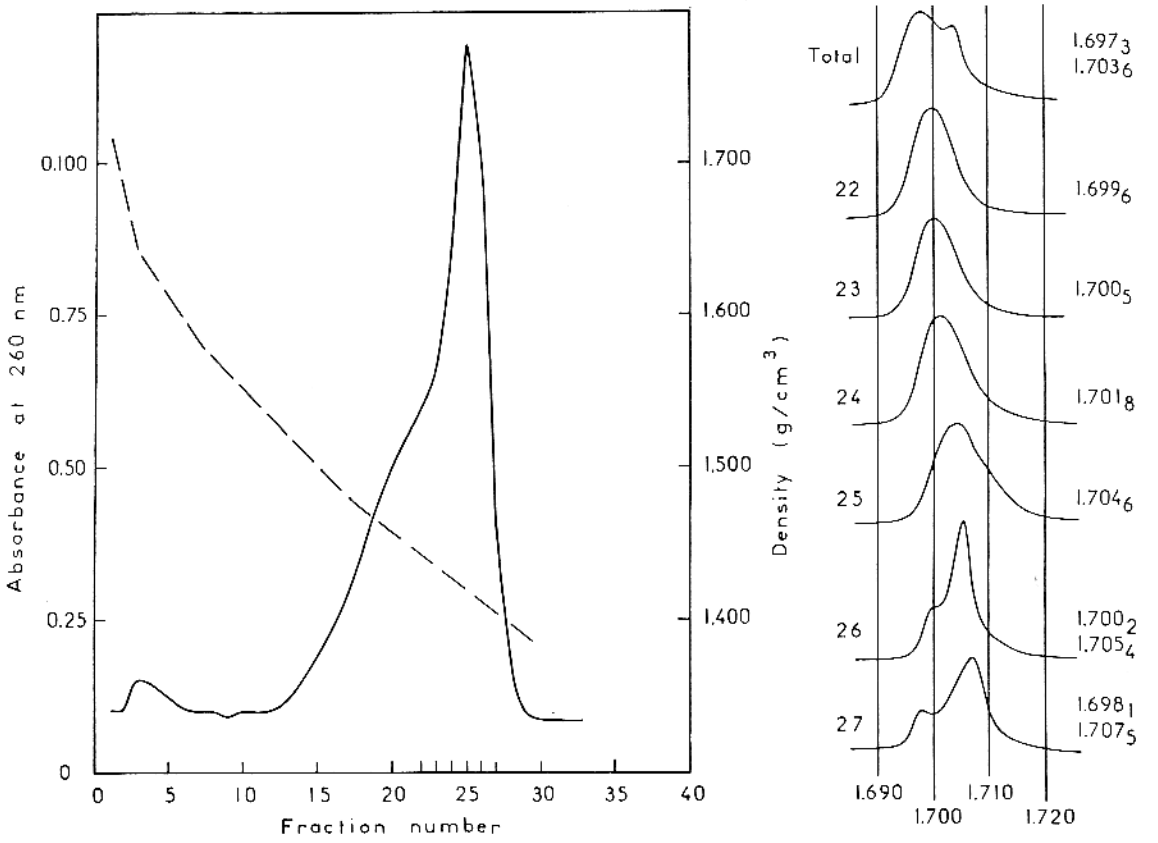

FIG. 3. — *Fractionation of* Cebus capucinus *DNA in Cs_2SO_4/BAMD gradient at R_f = 0.12.*
(a) (———) Absorbance profile of the centrifuged DNA solution (- - - -) density gradient.
(b) Analytical CsCl profiles of total DNA and individual fractions ; these had a volume of about 0.3 ml.

on all these DNA stands) were dark, but relatively less so in heterochromatic regions.

Preparation of C. capucinus *satellite DNA.*

Analytical centrifugation of *C. capucinus* DNA in CsCl density gradient (fig. 3b) revealed a main peak centered at 1.697_3 g/cm³ and a satellite peak at 1.703_6 g/cm³. Preparative centrifugation in BAMD/Cs_2SO_4 density gradient (fig. 3a) allowed the resolution of the satellite band which was obtained at about 80 per cent purity in fraction 26 ; in this fraction, the satellite band appeared as a sharp peak centered at 1.705_4 g/cm³. The satellite DNA appeared to band in the Cs_2SO_4/BAMD gradient between a 1.704 and a 1.708 g/cm³ component, as indicated by the analytical CsCl density gradient profiles of figure 3b.

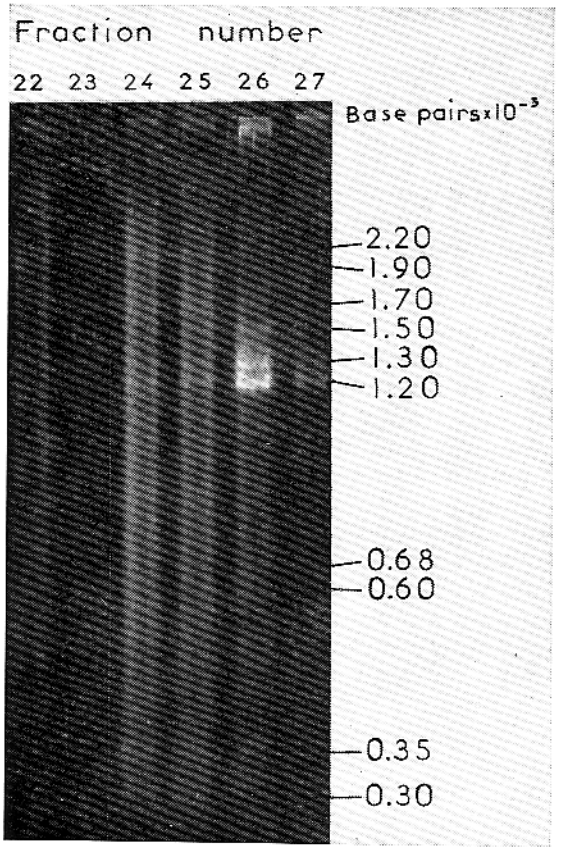

FIG. 4. — *Restriction endonuclease pattern of 6 individual fractions* (see figure 3 and text) : *DNAs were digested by HaeIII and electrophoresed on 6 per cent acrylamide gel.*

Degradation of fractions 24-27 of figure 4 by HaeIII, a restriction enzyme splitting the sequence GGCC, showed the presence of bands over a continuous smear ; the bands being particularly strong in fraction 26 (fig. 4). The molecular weights of the fragments seen on the gels indicated the existence of a multiplicity relationship among them.

In situ *hybridization.*

Hybridization of satellite-enriched fractions labeled with ^{32}P by nick-translation was found on all heterochromatic regions (fig. 5), grains being especially abundant on long segments carried by chromosomes 9, 11, 12, 13 and 18. The acridine orange staining after BrdU-incorporation made possible chromosomes identification and, on the other hand, sensitized to denaturation these regions of the karyotype which are otherwise the most resistant (T-positive bands). BrdU-incorporation appeared to be without effect on hybridization since no non-specific hybridization was detected on euchromatic regions having incorporated BrdU.

Discussion.

The establishment of clear correlation between the cytogenetic properties of heterochromatin and the base composition of the corresponding DNA is often made difficult by the small size or the ill-defined staining properties of the heterochromatic segments (such is the case for the secondary constrictions in human chromosomes). The case investigated here is an ideal one since the amount of heterochromatin in *C. capucinus* is very high (in fact, the highest reported so far in monkeys) and its cytogenetic properties well-defined. The latter all converge towards the conclusion that the heterochromatin of *C. capucinus* is dG+dC-rich.

(a) The large heterochromatin segments are T-positive and very resistant to heat treatment : in fact they are the only ones still stained after a long heating time, when the euchromatic R-bands have disappeared. This resistance to denaturation suggests a high dG+dC level.

(b) Quinacrine staining is dull. Although some exceptions are known, the correlation between dA+dT-richness and Q- band brightness is rather general [**22**, **36**]. Thus pale Q-bands observed suggest dA+dT-poorness.

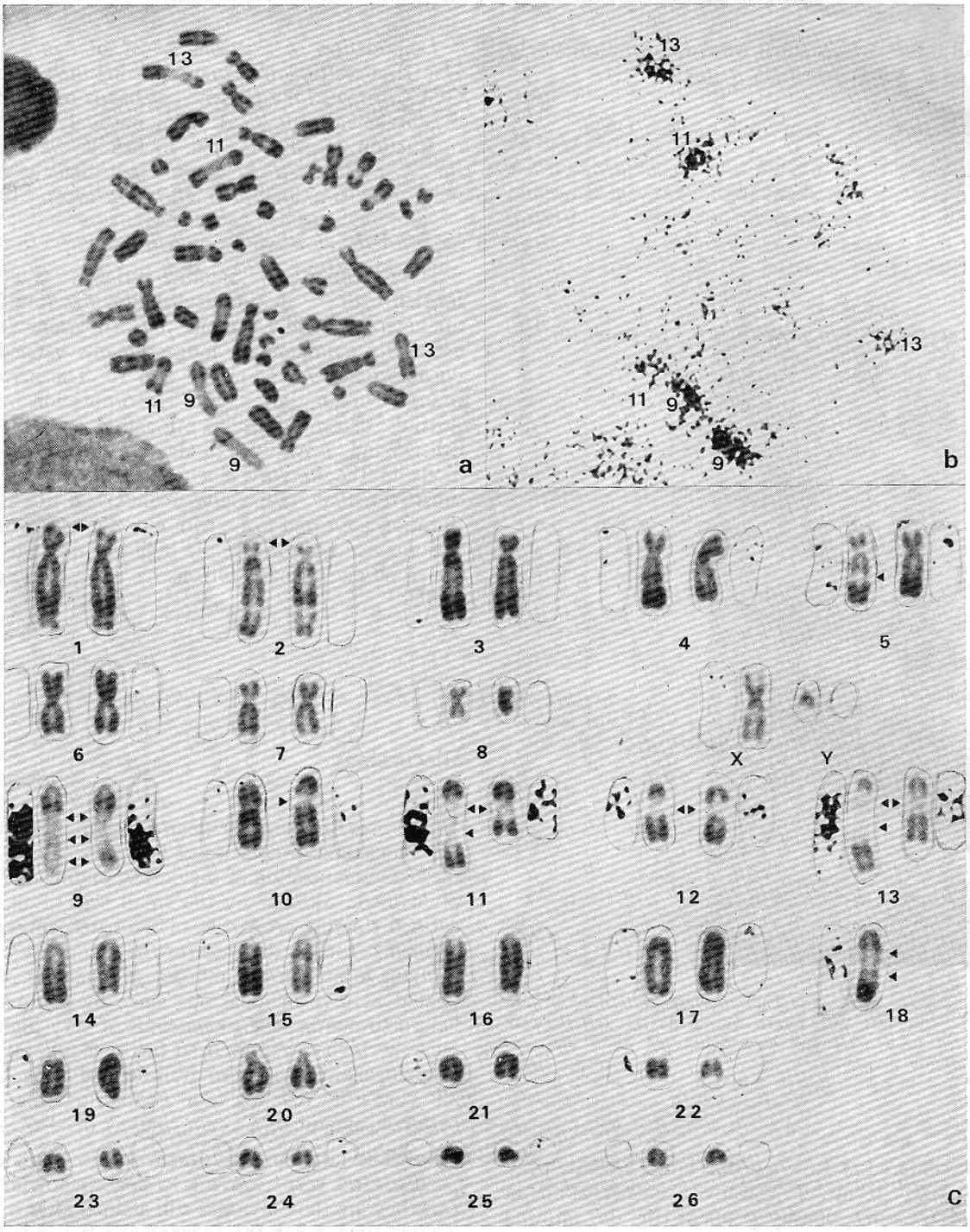

FIG. 5. — In situ *hybridization of* ^{32}P *satellite DNA.*

Chromosome banding was obtained by a 7-hour-BrdU-incorporation during the end of the last S-phase, followed by acridine orange staining. With such a treatment, the heterochromatic segments, being late replicating, are pale.

(a) Metaphase plate. The numbers of the chromosomes carrying the larger heterochromatic segments are indicated. In order the obtain a white background, a photographic countertype of the fluorescent picture was made.

(b) Autoradiography after *in situ* hybridization of the ^{32}P satellite DNA on the same metaphase plate.

(c) Karyotype made from the same metaphase plate, according to the classification given in reference 12. Grains are located on heterochromatic segments, T-positive in figure 1 (small triangles). There is an heteromorphism for these regions of chromosomes 11 and 13. A chromosome 18 was missing in this cell.

(c) After BrdU incorporation, the fluorescence of acridine orange-stained heterochromatin is always less quenched than that of the euchromatin. This indicates that BrdU incorporation is less pronounced in heterochromatin, implying a lower thymidine level compared to euchromatin.

(d) 5-azacytidine incorporation [37] leads to strong modifications of the heterochromatin segments, again indicating a dG+dC-richness. Because of the convergent indications derived from all above approaches, a dG+dC-rich satellite was looked for and found. Its relative amount, estimated at 13-15 per cent is in fair agreement with that of heterochromatin, as judged from relative chromosome segment length. Labelling with radioactive nucleotides followed by *in situ* hybridization confirmed the correspondence between the satellite sequences and the non-centromeric heterochromatic segments.

Similar, but more limited, results were previously obtained on chromosomes from different species having large heterochromatic segments with homogeneous staining properties. Three satellites, all of them dG+dC-rich, were localized at C-positive [6], G- or Q-negative [38] centromer regions of bovine autosomes. The same correlation was found for satellite from three different species [16]. In one of them it could be shown [19], in addition, that the heterochromatin belonged to two classes : G-positive terminal C-bands hybridizing dA+dT-rich satellite DNA, and G- or Q-negative centromer regions not hybridizing the dA+dT-rich satellite DNA, but probably containing dG+dC-rich satellite detected by density gradient centrifugation. dG+dC-rich satellites were also found in antelop squirrels whose chromosomes contain C-positive, G-negative heterochromatin [20]. To our knowledge, the present work is the first one, however, to demonstrate a correlation between R-positive, and not only G-negative, heterochromatin and dG+dC-rich satellite DNA.

In conclusion, it is evident that many factors play a role in the cytogenetic properties of heterochromatins. These include partial or total denaturation of chromatin, the interaction of dyes with chromosomal proteins, and the sequence-dependence of dye binding by DNA ; this latter factor is particularly important for the short repeated sequences of satellite DNAs. These factors account for unpredicted responses, particularly in the case of quinacrine mustard fluorescence [39]. However, when several cytogenetical techniques based on different principles, such as denaturation, dye binding, and base analog incorporation, are used together it appears possible to make correct predictions as to the base composition of satellite DNAs present in the heterochromatic regions of chromosomes.

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